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(21) International Application Number: PCT/US99/25666 (22) International Filing Date: 3 November 1999 (03.11.99) (30) Priority Data: 09/185,143 3 November 1998 (03.11.98) US (71) Applicant (for all designated States except US): PLANT GENETIC SYSTEMS NV [BE/BE]; Jozef Plateastraat 22, B-9000 Ghent (BE). (72) Inventors; and (75) Inventors/Applicants (for US only): MICHIELS, Frank [BE/BE]; Poelstraat 291, B-9820 Bottelare (BE). JOHNSON, Kirk [US/US]; 3926 Yana Place, Davis, CA 95616 (US). (74) Agents: LAWRENCE, William, F. et al.; Frommer Lawrence & Haug LLP, 745 Fifth Avenue, New York, NY 10151 (US).		(81) Designated States: AE, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CR, CU, CZ, DE, DK, DM, EE, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MA, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, TZ, UA, UG, US, UZ, VN, YU, ZA, ZW, ARIPO patent (GH, GM, KE, LS, MW, SD, SL, SZ, TZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GW, ML, MR, NE, SN, TD, TG). Published <i>With international search report.</i> <i>Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments.</i>
(54) Title: GLUFOSINATE TOLERANT RICE (57) Abstract This invention pertains to rice plants, plant material and seeds characterized by harboring a specific transformation event particularly by the presence of the <i>bar</i> gene under control of a CaMV 35S promoter, at a specific location in the rice genome. The rice plants of the invention combine glufosinate tolerance with optimal overall agronomic performance, genetic stability and adaptability to different genetic backgrounds.		

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Title of the invention**Glufosinate Tolerant Rice**5 Field of the invention

This invention pertains to rice plants, plant material and seeds characterized by harboring a specific transformation event particularly by the presence of the *bar* gene under control of a CaMV 35S promoter, at a specific location in the rice
10 genome. The rice plants of the invention combine glufosinate tolerance with optimal overall agronomic performance, genetic stability and adaptability to different genetic backgrounds.

All documents cited herein are hereby incorporated herein by reference.

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Background of the invention

The phenotypic expression of a transgene in a plant is determined both by the structure of the gene itself and by its location in the plant genome. At the same time
20 the presence of the transgene at different locations in the genome will influence the overall phenotype of the plant. The agronomically or industrially successful introduction of a commercially interesting trait in a plant by genetic manipulation can be a lengthy procedure dependent on different factors. The actual transformation and regeneration of genetically transformed plants are only the first in a series of selection
25 steps which include extensive genetic characterization, breeding, and evaluation in field trials.

Rice production is commonly threatened by various weeds. Some of these can be highly competitive and in cases of severe infestation can result in yield loss of such
30 magnitude that it makes the crop economically unattractive. For direct-seeded, mechanized rice cultivation typical of temperate production, both cultural practices (e.g. crop rotation, irrigation management) and herbicides are necessary to control weeds (Hill et al. 1994).

The *bar* gene (Thompson et al, 1987, EMBO J. 6:2519-2523; Deblock et al. 1987, EMBO J. 6:2513-2518) is a gene encoding the enzyme phosphinothricin acetyl transferase (PAT), which, when expressed in a plant, confers resistance to the herbicidal compounds phosphinothricin (also called glufosinate) or bialaphos (see also for example US patents, 5,646,024 and 5,561,236) and salts and optical isomers thereof. Other genes encoding PAT have been described (see for example: Wohlleben et al., 1988, Gene 70:25-37; EP 275,957; US 5,276,268; US 5,637,489; US 5,273,894). The transformation of monocotyledonous plants by electroporation of intact tissue capable of forming compact embryogenic callus or compact embryogenic callus obtained from such tissue is described in US Patent 5,641,664. Herein, transformation of compact embryogenic callus of rice by electroporation of a *bar* gene and the regeneration of transgenic rice plants is disclosed.

Transgenic rice plants containing the *gus* gene with either the *bar* gene, or the *hyg* gene conferring resistance to hygromycin, obtained by the transformation of cells of immature rice embryos by bombardment with DNA-coated gold particles have been described (Christou et al, 1991: Biotechnology 9:957).

Transformation of rice with the *bar* gene by electroporation of aggregated suspension cells is described in US Patent 5,679,558.

However, the foregoing documents fail to teach or suggest the present invention.

Summary of the invention

The present invention relates to a transgenic, glufosinate tolerant rice plant, cell, tissue or seed, which is characterized by one or both of the following characteristics:

- a) the genomic DNA of said plant, cell, tissue or seed is capable of yielding at least one, or advantageously at least two, preferably at least three, for instance at least four, more preferably five of the sets of restriction fragments, selected from the group of:

- i) one set of NsiI fragments comprising at least: one fragment with a length between about 5077 and about 14057 bp, preferably of about 12 kbp and one with a length between about 5077 and about 11497 bp, preferably of about 7,0 kbp;
- 5 ii) one set of NcoI fragments comprising at least: one fragment with a length between about 2838 and about 4507 bp, preferably of about 3,2 kbp, one fragment with a length between about 2140 and about 2443 bp, preferably of about 2,3 kbp, and one fragment with a length between about 1986 and about 2140 bp, preferably of about 2,1 kbp; preferably also comprising one fragment with a length between about 805 and about 1093 bp, preferably of about 1,0 kbp;
- 10 iii) one set of HindIII fragments comprising at least: one fragment with a length of more than about 11497 bp, preferably of about 14 kbp, and one fragment with a length between about 5077 and about 14057 bp, preferably of about 13 kbp;
- iv) one set of EcoRV fragments comprising at least: one fragment with a length between about 1700 and about 1986 bp, preferably of about 1,7 kbp, one fragment with a length between about 1159 and about 1700 bp, preferably of about 1,6 kbp, and one fragment with a length between about 805 and about 1093 bp, preferably of about 1,0 kbp; preferably also comprising one or more of the following: one fragment with a length between about 5077 and about 14057 bp, preferably of about 12 kbp, one fragment with a length between about 4507 and about 5077 bp, preferably of about 4,7 kbp, one fragment with a length between about 2838 and about 4507 bp, preferably of about 2,9 kbp, one fragment with a length between about 805 and about 1159 bp, preferably of about 1,1 kbp, and one fragment with a length of between about 514 and about 805 bp, preferably of about 600 bp;
- 15 20 v) one set of EcoRI fragments preferably comprising at least: one fragment with a length between about 1159 and about 1986 bp, preferably of about 1,7 bp, and one fragment with a length between about 1159 and about 1700 bp, preferably of about 1327 bp; preferably also comprising one fragment with a length between about 514 and 805 bp, preferably of about 0,7 kbp, and one fragment with a length of less than about 805 bp, preferably of about 0,5 kbp;
- 25 30 wherein each of the restriction fragments is capable of hybridizing under standard stringency conditions, with the about 1327 bp fragment obtainable by EcoRI digestion of the plasmid having the nucleotide sequence of SEQ ID No 6; and/or,

b) the genomic DNA of the plant, cell, tissue or seed can be used to amplify a DNA fragment of between about 490 and about 550 bp, preferably of about 522 bp using a polymerase chain reaction with two primers having the nucleotide sequence of SEQ ID No 2 and SEQ ID No 3 respectively (or includes a DNA fragment of about 490 to about 550 bp, preferably of about 522 bp amplified using a polymerase chain reaction with two primers having the nucleotide sequence of SEQ ID No 2 and SEQ ID No 3 respectively).

The present invention relates to a transgenic, glufosinate tolerant rice plant, cell, tissue or seed, which is characterized in that the genomic DNA of the plant, cell, tissue or seed is capable of yielding at least one, advantageously at least two, preferably at least three, for instance at least four, more preferably five sets of restriction fragments selected from the group described above comprising the sets of restriction fragments described under i), ii), iii), iv) and v) above, whereby the selection can include any combination of i), ii), iii), iv) and v) described above.

The present invention relates to a transgenic, glufosinate tolerant rice plant, cell, tissue or seed which is preferably characterized by both of the characteristics described under a) and b) above.

The invention also relates to the seed deposited at the ATCC under number ATCC 203353, a plant which is grown from this seed, and cells or tissues from a plant grown from this seed. The invention further relates to plants obtainable by propagation of, and/or breeding with a rice plant grown from the seed deposited at the ATCC under number ATCC 203353.

The invention further relates to plants, seeds, cells or tissues (e.g., rice plants, seeds, cells or tissues) comprising herein discussed flanking regions with the 35S-bar gene (as herein discussed) therebetween, or plants, seeds, cells, or tissues (e.g., rice plants, seeds, cells or tissues) comprising a nucleotide sequence which is at least 65%, e.g., at least 75%, such as at least 80%, for instance at least 85%, such as at least 90%, for example at least 95% or even 97% or 100% similar to a sequence disclosed herein,

such as the sequence for the flanking region-35S-bar gene-flanking region construct, or the insertion region.

The invention further relates to a process for cultivating rice plants of the invention as described above, more preferably a process which comprises applying a herbicide with glufosinate as an active ingredient to the cultivated rice plants.

It is believed that the rice plants of the invention, when cultivated according to the process described above, which comprises applying a herbicide with glufosinate as an active ingredient, display improved growth as compared to untransformed rice of the same cultivar (US 5,739,082). Thus, the invention can comprehend a method for improving the yield or growth of rice plants.

The invention also provides a process for breeding rice which comprises a crossing with the rice plants of the invention.

The invention further provides a process for producing a transgenic cell of a rice plant or a plant obtained therefrom, which comprises inserting a recombinant DNA molecule into a part of the chromosomal DNA of a rice cell characterized by the sequence of SEQ ID No 7 and, optionally, regenerating a rice plant from the transformed rice cell.

The invention can further include a nucleotide sequence which is at least 65%, e.g., at least 75%, such as at least 80%, for instance at least 85%, such as at least 90%, for example at least 95% or even 97% or 100% similar to a sequence disclosed herein.

The invention further relates to a method for identifying a transgenic plant, or cells or tissues thereof, which method comprises establishing one or both of the following characteristics of the genomic DNA of the transgenic plant, or its cells or tissues:

- a) the genomic DNA is capable of yielding at least two, preferably at least three, particularly at least 4, more particularly five of the sets of restriction fragments, wherein selected from the group of:

- i) one set of NsiI fragments wherein one fragment has a length between 5077 and 14057 bp, preferably of about 12 kbp and one has a length between 5077 and 11497 bp, preferably of about 7,0 kbp;
- ii) one set of NcoI fragments wherein one fragment has a length between 2838 and 4507 bp, preferably of about 3,2 kbp, one fragment has a length between 2140 and 2443 bp, preferably of about 2,3 kbp, one fragment has a length between 1986 and 2140 bp, preferably of about 2,1 kbp, and one fragment has a length between 805 and 1093 bp, preferably of about 1,0 kbp;
- iii) one set of HindIII fragments wherein one fragment has a length of more than 11497 bp, preferably of about 14kbp, and one fragment has a length between 5077 and 14057 bp, preferably of about 13 kbp;
- iv) one set of EcoRV fragments wherein one fragment has a length between 5077 and 14057 bp, preferably of about 12 kbp, one fragment has a length between 4507 and 5077 bp, preferably of about 4,7 kbp, one fragment has a length between 2838 and 4507 bp, preferably of about 2,9 kbp, one fragment has a length between 1700 and 1986 bp, preferably 1,7 kbp, one fragment has a length between 1159 and 1700 bp, preferably of about 1,6 kbp, one fragment has a length between 805 and 1159 bp, preferably of about 1,1 kbp, one fragment has a length between 805 and 1093 bp, preferably of about 1,0 kbp, and one fragment has a length of between 514 and 805 bp, preferably of about 600 bp;
- v) one set of EcoRI fragments, wherein one fragment has a length between 1159 and 1986 bp, preferably of about 1,7 bp, one fragment has a length between 1159 and 1700 bp, preferably of about 1327 bp, one has a length between 514 and 805 bp, preferably about 0,7 kbp, and one fragment has a length of less than 805 bp, preferably of about 0,5 kbp;

wherein each of the restriction fragments is capable of hybridizing under standard stringency conditions, with the 1327 bp fragment obtainable by EcoRI digestion of the plasmid having the nucleotide sequence of SEQ ID No. 6; and/or,

- b) the genomic DNA of the plant, cell, tissue or seed can be used to amplify a DNA fragment of about 522 bp using a polymerase chain reaction with two primers having the nucleotide sequence of SEQ ID No. 2 and SEQ ID No. 3 respectively.

The invention further relates to a kit for identifying the transgenic plants comprising the elite event of the present invention, said kit comprising PCR probes recognizing the T-DNA and the 3' or 5' flanking sequence of GAT-OS1, preferably having the nucleotide sequence of SEQ ID No. 2 and SEQ ID No. 3 respectively, for use in the PCR identification protocol.

Brief description of the drawings

The following detailed description, given by way of example, but not intended to limit the invention to specific embodiments described, may be understood in conjunction with the accompanying Figures, incorporated herein by reference, in which:

Fig. 1. Restriction map obtained after digestion of GAT-OS1 genomic DNA

Loading sequence of the gel analyzed by Southern blot: lane 1, non-transgenic rice DNA, lane 2, Control plasmid DNA digested with EcoRI, lane 3, GAT-OS1 DNA digested with NsiI, lane 4, GAT-OS1 DNA digested with NcoI, lane 5, GAT-OS1 DNA digested with HindIII, lane 6, GAT-OS1 DNA digested with EcoRV, lane 7, GAT-OS1 DNA digested with EcoRI, lane 8, Lambda DNA digested with PstI.

Fig. 2. PCR analysis of different lines using the GAT-OS1 PCR identification protocol.

Loading sequence of the gel: lane 1, molecular weight marker (100bp ladder), lanes 2 to 11, DNA samples from rice plants comprising different transgenic events, lane 12, DNA from M202 wild-type, lane 13, DNA from Bengal wild-type, lane 14, negative control (water), lane 15, molecular weight marker (100bp ladder).

Detailed description

The term "gene" as used herein refers to any DNA sequence comprising several operably linked DNA fragments such as a promoter and a 5' untranslated region (the 5'UTR), which together form the promoter region, a coding region (which may or may not code for a protein), and an untranslated 3' region (3'UTR) comprising a polyadenylation site. Typically in plant cells, the 5'UTR, the coding region and the 3'UTR are transcribed into a RNA which, in the case of a protein encoding gene, is translated into the protein. A gene may include additional DNA fragments such as, for

example, introns. As used herein, a genetic locus is the position of a given gene in the genome of a plant.

The term "chimeric" when referring to a gene or DNA sequence is used to refer to the fact that the gene or DNA sequence comprises at least two functionally relevant DNA fragments (such as promoter, 5'UTR, coding region, 3'UTR, intron) that are not naturally associated with each other and originate, for example, from different sources. "Foreign" referring to a gene or a DNA sequence with respect to a plant species is used to indicate that the gene or DNA sequence is not naturally found in that plant species.

As used herein the term "transgene" refers to a recombinant DNA molecule as incorporated in the genome of a plant. The term "recombinant DNA molecule" is used to exemplify and thus can include an isolated nucleic acid molecule which can be DNA and which can be obtained through recombinant or other procedures. This recombinant DNA molecule usually comprises at least one copy of at least one "gene of interest" (e.g. a chimeric gene) which is capable of conferring one or more specific characteristics to the transformed plant. A "transgenic plant" refers to a plant comprising a transgene in the genome of all of its cells.

The incorporation of a recombinant DNA molecule in the plant genome typically results from transformation of a cell or tissue (or from another genetic manipulation). The particular site of incorporation is either due to chance or is at a predetermined location (if a process of targeted integration is used).

The transgene can be characterized by the location and the configuration at the site of incorporation of the recombinant DNA molecule in the plant genome. The site in the plant genome where a transgene has been inserted is also referred to as the "insertion site" or "target site". Insertion of the transgene into the plant genome can be associated with a deletion of plant DNA, referred to as "target site deletion". A "flanking region" or "flanking sequence" as used herein refers to a sequence of at least 20 bp, preferably at least 50 bp, and up to 5000 bp of the plant genome which is located either immediately upstream of and contiguous with or immediately

downstream of and contiguous with the transgene. Transformation procedures leading to random integration of the transgene will result in transformants with different flanking regions, which are characteristic and unique for each transformant. When the transgene is introduced into a plant through traditional crossing, its insertion site in the plant genome, or its flanking regions will generally not be changed. An "insertion region" as used herein refers to the region corresponding to the region encompassed by at least one of the flanking regions of a transgene in the (untransformed) plant genome.

10 Expression of the transgene is used to indicate that the gene(s) of interest comprised in the transgene is expressed so as to confer on the plant one or more phenotypic traits (e.g. herbicide tolerance) that were intended to be conferred by the introduction of the recombinant DNA molecule - the transforming DNA - used during transformation (on the basis of the structure and function of part or all of the gene(s) of interest).

15 An event is defined as a (artificial) genetic locus that, as a result of genetic manipulation, carries a transgene comprising at least one copy of a gene of interest. The typical allelic states of an event are the presence or absence of the transgene. An event is characterized phenotypically by the expression of the transgene. At the genetic level, an event is part of the genetic makeup of a plant. At the molecular level, an event is characterized by the restriction map (e.g. as determined by Southern blotting) and/or by the upstream and/or downstream flanking sequences of the transgene, and/or the molecular configuration of the transgene. Usually transformation of a plant with a transforming DNA comprising at least one gene of interest leads to a multitude of events, each of which is unique.

25 An elite event, as used herein, is an event which is selected from a group of events obtained by transformation with the same transforming DNA, based on the expression and stability of the transgene and its compatibility with optimal agronomic characteristics of the plant comprising it. Thus the criteria for elite event selection are one or more, preferably two or more, advantageously all of the following:

- a) That the presence of the transgene does not compromise other desired characteristics of the plant, such as those relating to agronomic performance or commercial value;
- b) That the event is characterized by a well defined molecular configuration which is stably inherited and for which appropriate diagnostic tools for identity control can be developed;
- c) That the gene(s) of interest in the transgene shows a correct, appropriate and stable spatial and temporal phenotypic expression, both in heterozygous (or hemizygous) or homozygous condition of the event, at a commercially acceptable level in a range of environmental conditions in which the plants carrying the event are likely to be exposed in normal agronomic use;

It is preferred that the transgene is associated with a position in the plant genome that allows introgression into desired commercial genetic backgrounds.

15

The status of an event as an elite event is confirmed by introgression of the elite event in different relevant genetic backgrounds and observing compliance with one, two or all of the criteria, e.g., a), b) and c) above.

- 20 An "elite event" thus refers to a genetic locus comprising a transgene, which answers to the above-described criteria. A plant, plant material or progeny such as seeds can comprise the elite event in its genome.

25 The "diagnostic tools" developed to identify an elite event or the plant or plant material comprising an elite event, are based on the specific genomic characteristics of the elite event, such as, a specific restriction map of the genomic region comprising the transgene and/or the sequence of the flanking region(s) of the transgene. A "restriction map" as used herein refers to a set of Southern blot patterns obtained after cleaving plant genomic DNA with a particular restriction enzyme, or set of restriction enzymes, and hybridization with a probe sharing sequence similarity with the transgene (under specific conditions). Due to the (endogenous) restriction sites present in a plant genome prior to incorporation of the transgene, insertion of a transgene will alter the specific restriction map of that genome. Thus, a particular

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transformant or progeny derived thereof can be identified by one or more specific restriction patterns. The conditions for determining the restriction map of an event are laid out in a restriction map identification protocol.

5 Alternatively, plants or plant material comprising an elite event can be identified by testing according to a PCR identification protocol. This is a PCR using primers which specifically recognize the elite event. Essentially, a set of primers is developed which recognizes a) a sequence within the 3' or 5' flanking sequence of the elite event and b) a sequence within the foreign DNA, which primers amplify a fragment (integration
10 fragment) preferably of between 100 and 350 nucleotides. Preferably, a control is included of a set of primers which amplifies a fragment within a housekeeping gene of the plant species (preferably a fragment which is larger than the amplified integration fragment). The optimal conditions for the PCR, including the sequence of the specific primers is specified in a PCR identification protocol.

15

The term "similarity", for instance, with respect to a nucleotide sequence, is intended to indicate a quantitative measure of homology between two sequences. The percent sequence similarity can be calculated as

$(N_{ref} - N_{dif}) * 100 / N_{ref}$, wherein N_{dif} is the total number of non-identical residues in
20 the two sequences when aligned and wherein N_{ref} is the number of residues in one of the sequences. Hence, the DNA sequence AGTCAGTC will have a sequence similarity of 75% with the sequence AATCAATC

($N_{ref} = 8$; $N_{dif} = 2$). The invention comprehends nucleic acid molecules and with sequences having at least 65%, e.g., at least 70%, such as at least 75%, or at least 80%
25 or advantageously at least 85%, for instance at least 90%, such as at least 95% or even 97% or 100% similarity with sequences disclosed herein, as well as plants, cells, tissues, seeds, and progeny thereof (e.g., rice plants, cells, tissues, seeds and progeny thereof) comprising such nucleic acid molecules. Alternatively or additionally, "similarity" with respect to sequences refers to the number of positions with identical
30 nucleotides divided by the number of nucleotides in the shorter of the two sequences wherein alignment of the two sequences can be determined in accordance with the Wilbur and Lipmann algorithm (Wilbur and Lipman, 1983 PNAS USA 80:726) using a window size of 20 nucleotides, a word length of 4 nucleotides, and a gap penalty of

4, and computer-assisted analysis and interpretation of the sequence data including alignment can be conveniently performed using programs of the Intelligenetics™ Suite (Intelligenetics Inc. CA). Sequences which are "essentially similar" have a sequence similarity or identity of at least about 75%, advantageously at least about 80%, such as at least about 85%, preferably at least about 90%, especially about 95%, such as at least 97%, and especially about 100%. It is clear that when RNA sequences are said to be essentially similar or similar, or have a degree of sequence identity with DNA sequences, thymidine (T) in the DNA sequence is considered equal to uracil (U) in the RNA sequence.

10

The present invention relates to the development of an elite event in rice, GAT-OS1, and the plants, plant cells, or plant material derived from this event. Plants comprising elite event GAT-OS1 were obtained through transformation with a 1501 bp PvuI-HindIII fragment of plasmid pB5/35Sbar (SEQ ID No. 6) as described in example 1.

15

The recombinant DNA molecule used for generation of this elite event comprises a DNA sequence encoding the enzyme phosphinothricin acetyl transferase and the 35S promoter of Cauliflower Mosaic Virus, wherein the sequence encoding phosphinothricin acetyl transferase is under the control of the 35S promoter of Cauliflower Mosaic Virus (termed the "35S-bar gene"). The 35S promoter has a "constitutive" expression pattern in rice (Battraw et al, 1990, Plant Mol Biol 15:527-538), which means that it is significantly expressed in most plant cell types, during most of the plant life cycle. The expression of the 35S-bar gene in rice plants confers resistance to the herbicidal compounds phosphinothricin or bialaphos or glufosinate or more generally, glutamine synthetase inhibitors, or salts or optical isomers thereof.

25

Plants or plant material comprising GAT-OS1 can be identified according to the restriction map identification protocol described in Example 3b)(1) herein. Briefly, rice genomic DNA is digested with a selection (preferably one or more such as two to five) of the following restriction enzymes: NsiI, NcoI, HindIII, EcoRV, EcoRI, is then transferred to nylon membranes and hybridized with the about 1327 bp EcoRI fragment of plasmid pB5/35Sbar. It is then determined for each restriction enzyme used whether the following fragments can be identified:

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- NsiI: at least one fragment of between about 5077 and about 14057 bp, preferably of about 12 kbp, and one fragment of between about 5077 and about 11497 bp, preferably of about 7,0 kbp
- NcoI: at least one fragment of between about 2838 and about 4507 bp, preferably of about 3,2 kbp, one fragment of between about 2140 and about 2443 bp, preferably of about 2,3 kbp, and one fragment of between about 1986 and about 2443 bp, preferably of about 2,1 kbp; preferably also one fragment of between about 805 and about 1093 bp, preferably of about 1,0 kbp
- HindIII: at least one fragment of more than about 11497 bp, preferably of about 14kbp, and one fragment of between about 5077 and about 14057 bp, preferably of about 13 kbp
- EcoRV: at least one fragment of between about 1700 and about 1986 bp, preferably of about 1,7 kbp, one fragment of between about 1159 and about 1700 bp, preferably of about 1,6 kbp, one fragment of between about 805 and about 1093 bp, preferably of about 1,0 kbp; preferably also one or more of the following: one fragment of between about 5077 and about 14057 bp, preferably of about 12 kbp, one fragment of between about 4507 and about 5077 bp, preferably of about 4,7 kbp, one fragment of between about 2838 and about 4507 bp, preferably of about 2,9 kbp, one fragment of between about 805 and about 1159 bp, preferably of about 1,1 kbp, and one fragment of between about 514 and about 805 bp, preferably of about 600 bp
- EcoRI: at least one fragment of between about 1159 and about 1986 bp, preferably of about 1,7 bp, and one fragment of between about 1159 and about 1700 bp, preferably of about 1327 bp; preferably also one or both of: one fragment of between about 514 and about 805 bp, preferably of about 0,7 kbp, and one fragment of less than about 805 bp, preferably of about 0,5 kbp.

The lengths of the DNA fragments are determined by comparison with a set of DNA fragments of known length, preferably the PstI fragments of phage lambda DNA.

If the plant material after digestion with one or more, such as at least two, preferably at least 3, for instance with at least 4, more preferably with all of these restriction

enzymes, yields DNA fragments with the same length as those described above, the rice plant is determined to harbor elite event GAT-OS1.

Plants or plant material comprising GAT-OS1 can also be identified according to the
5 PCR identification protocol described in Example 3b)(2) herein. Briefly, rice genomic DNA is amplified by PCR using a primer which specifically recognizes a flanking sequence of GAT-OS1, preferably the primer with the sequence of SEQ ID No 3, and a primer which recognizes a sequence in the transgene, preferably the primer with the sequence of SEQ ID No 2. Endogenous rice primers are used as controls. If the plant
10 material yields a fragment of between about 490 and about 550 bp, preferably of about 522 bp, the rice plant is determined to harbor elite event GAT-OS1.

Plants harboring GAT-OS1 are also characterized by their glufosinate tolerance, which in the context of the present invention includes that plants are tolerant to the
15 herbicide LibertyTM. Tolerance to LibertyTM is defined by the criterium that spraying of the plants in the three to four leaf stage (3V to 4V) with at least 200 grams active ingredient/hectare (g.a.i./ha), preferably 400 g.a.i./ha, and possibly up to 1600 g.a.i./ha, does not kill the plants.

20 Plants harboring GAT-OS1 are of course further characterized by the presence in their cells of phosphinothricin acetyl transferase as determined by a PAT assay (De Block et al, 1987, supra).

Plants harboring GAT-OS1 can, for example, be obtained from seeds deposited at the
25 ATCC under number ATCC 203353. Such plants can be further propagated and/or used in a conventional breeding scheme to produce more transformed plants with the same characteristics or to introduce the elite event of the invention into other cultivars of the same plant species. Seeds obtained from these plants contain the elite event stably incorporated into their genome.

30

The rice plants of this invention can be cultivated in a conventional way. The presence of the transgene ensures that they are tolerant to glufosinate. Therefore,

weeds in the fields where such rice plants are grown can be controlled by application of herbicides comprising glufosinate as an active ingredient (such as LibertyTM).

Plants harboring GAT-OS1 are also characterized by having agronomical characteristics which are comparable to the following commercially available rice varieties in the US: M202, M201, M103, Drew, Kaybonnet, Lagrue, Priscilla, Cypress, Bengal, Cocadrie, Jefferson, Madison. The agronomical characteristics of relevance are: plant height, strength/stiffness of straw, resistance to lodging, leaf morphology (length, width and angle for flag leaf), time to maturity, floret confirmation, panicle fertility, complete closure of the hull on the seed, grain size and shape, and grain production and yield.

It has been observed that the presence of the transgene in this region of the rice plant genome, more preferably at this site of the rice plant genome, confers particularly interesting phenotypic and molecular characteristics to this event. More specifically, the presence of a transgene at this particular site in the genome results in stable phenotypic expression of the transgene without significantly compromising any aspect of desired agronomic performance of the plant. Thus, the insertion region is shown to be particularly suited for the introduction of a gene(s) of interest, such as a herbicide resistance gene, more specifically a gene encoding phosphinothricin acetyl transferase under the control of a 35S promoter, particularly the PvuI-HindIII fragment of plasmid pB5/35Sbar.

A recombinant DNA molecule can be specifically inserted in this insertion region by targeted insertion methods. Such methods are well known to those skilled in the art and comprise, for example, homologous recombination using a recombinase such as, but not limited to either FLP recombinase from *Saccharomyces cerevisiae* (US Patent 5,527,695), the CRE recombinase from *Escherichia coli* phage P1 (published PCT application WO 9109957, the recombinase from pSRI of *Saccharomyces rouxii* (Araki et al. 1985, J Mol Biol 182:191-203), or the lambda phage recombination system such as described in US Patent 4,673,640.

DNA can be inserted into a plant genome, such as a rice genome by techniques including, electroporation methods, bombardment with DNA-coated gold particles or biolistic methods, or agrobacterium or polyethylene glycol mediated methods, and the like.

5

As used herein "comprising" is to be interpreted as specifying the presence of the stated features, integers, steps or components as referred to, but does not preclude the presence or addition of one or more features, integers, steps or components, or groups thereof. Thus, e.g., a nucleic acid or protein comprising a sequence of nucleotides or amino acids, may comprise more nucleotides or amino acids than the actually cited ones, i.e., be embedded in a larger nucleic acid or protein. A chimeric gene comprising a DNA sequence which is functionally or structurally defined, may comprise additional DNA sequences, etc.

15 The following examples describe the development and characteristics of rice plants harboring the elite event GAT-OS1.

Unless otherwise stated, all recombinant DNA techniques are carried out according to standard protocols as described in Sambrook et al. (1989) *Molecular Cloning: A Laboratory Manual*, Second Edition, Cold Spring Harbour Laboratory Press, NY and in Volumes 1 and 2 of Ausubel et al. (1994) *Current Protocols in Molecular Biology*, Current Protocols, USA. Standard materials and methods for plant molecular work are described in Plant Molecular Biology Labfax (1993) by R.D.D. Croy published by BIOS Scientific Publications Ltd (UK) and Blackwell Scientific Publications, UK.

25

In the description and examples, reference is made to the following sequences:

SEQ ID No. 1:	sequence comprising the 3' flanking region of GAT-OS1
SEQ ID No. 2:	OSA03: primer of the PCR identification protocol
30 SEQ ID No. 3:	OSA05: GAT-OS1-specific primer of the PCR identification protocol
SEQ ID No. 4:	OSA01: rice endogenous primer
SEQ ID No. 5:	OSA02: rice endogenous primer

SEQ ID No. 6: plasmid pB5/35Sbar
 SEQ ID No. 7: insertion region

EXAMPLES

5 Example 1. Transformation of rice with a gene encoding herbicide resistance

a) Construction of the chimeric DNA comprising the bar gene under the control of a 35S promoter (pB5/35Sbar).

10 A plasmid pB5/35Sbar was constructed following standard procedures. The sequence of plasmid pB5/35Sbar is given in SEQ ID No. 6. Digestion with PvuI-HindIII yielded a 1501 bp fragment which comprised the following genetic elements:

Nucleotide coordinates	Genetic elements
2140-2195	Sequence derived from pUC19 (Yanish-Perron et al., Gene 33:103-119, 1985)
2196-2204	Synthetic polylinker sequence
2205-2398	Complement of 35S terminator (T35S) from Cauliflower Mosaic Virus (Franck A. et al., Cell 21:285-294, 1980; Pietrzak M. et al., Nucl. Acids Res. 14:5857-5868, 1986)
2399-2417	Synthetic polylinker sequence
2418-2969	Complement of bar gene from <i>Streptomyces hygroscopicus</i> (Thompson et al., EMBO J. 6:2519-2523, 1986)
2970-2985	Synthetic polylinker sequence
2986-3517	Complement of 35S promoter (P35S) from Cauliflower Mosaic Virus (Franck et al. above; Pietrzak et al., above)
3518-3641	Sequence derived from pUC19, (Yanisch-Perron et al., above)

15 The 1501 bp PvuI-HindIII fragment was purified by extraction of this fragment after electrophoresis.

b) Transformation of rice

The variety M-202 is a medium grain rice developed in the California Co-operative Rice Research Foundation. It is a pure line selected from a cross made in 1977. Foundation seed was made available to growers in 1985. The pedigree includes IR-8, CS-MS, 10-7, M9 and M-101. M-101 was derived from a mutation in Calrose (Johnson C.W. et al. 1986, Crop Science 26:198).

Transformation of rice plants with the 1501 bp PvuI-HindIII fragment of pB5/35Sbar was performed using direct DNA transfer. Selection was done on phosphinothricin (PPT) at all stages except plantlet regeneration, which was done in the absence of PPT to accelerate growth. This resulted in a set of primary transformants (plants of generation T₀).

Example 2. Development of events

a) Development of transgenic homozygous lines

The various T₀ hemizygous plantlets were transitioned from tissue culture, transferred to greenhouse soil, and allowed to flower and set seed. Plantlets were evaluated for fertility, fecundity and tolerance to glufosinate ammonium. 20 plants were selected for further analysis. T₁ seed produced by selfing was collected from these plants and grown in the field. T₁ plants were sprayed with LibertyTM herbicide at 800 grams active ingredient per hectare (g.a.i./ha; recommended dosage for farmers 400 g.a.i./ha). The events that survived the herbicide application and segregated 3:1 for herbicide tolerance were selected for further evaluation. Tolerant plants were evaluated for damage (leaf tip burn).

T₂ seeds were harvested from panicles of all tolerant plants of selected events. These were sown in rows and T₂ plants were sprayed with LibertyTM herbicide (1600 g.a.i./ha) to evaluate segregation of the herbicide tolerance. Those rows that had 100% survivors and thus corresponded to lines which were homozygous for the transgene

were selected. These were again evaluated for herbicide damage and phenotypic traits. Further selection of events was made based on uniformity of phenotype within the panicle row (for the desired characteristics).

5 b) Characterization of transgenic events – selection of GAT-OS1

Transgenic events were further characterized for southern blot patterns, general phenotype and agronomic performance, and yield. Where appropriate these characteristics were determined in field conditions.

10

Southern blot analysis

Presence of the transgene was checked by standard Southern blot analysis using enzymatic digestion of rice genomic DNA with EcoRI or EcoRV and hybridization to
15 the 1327 bp EcoRI fragment of pB5/35Sbar. The relative band intensity provided an indication on whether plants were homozygous or hemizygous for the transgenic locus. All events except one were found to have simple insertions. This was confirmed by the fact that the segregation pattern of the transgene could be explained by Mendelian inheritance of a simple locus.

20

General plant phenotype and agronomic performance

T₁ and T₂ plants were evaluated for a number of phenotypic traits including plant height, strength/stiffness of straw, tendency to lodge, leaf morphology (too thin or
25 incorrect angle for flag leaf), late maturity, floret configuration, panicle sterility or incomplete fertility, incomplete closure of the hull on the seed (which would lead to increased disease susceptibility), grain size and shape, and grain production and yield. Lines were evaluated to be similar (or improved) in displayed agronomic characteristics compared to the untransformed M202 cultivar and the following rice
30 varieties: M201, M103, Drew, Kaybonnet, Lagrue, Priscilla, Cypress, Bengal, Cocadrie, Jefferson, Madison. In some cases, the plants within a panicle row segregated for somaclonal variation for one or more of the above-mentioned traits.

Unless this resulted in the introduction of a commercially interesting phenotypic trait, these plants were discarded.

Field trials for yield evaluation

5

T2 seeds were harvested in bulk from the selected homozygous populations and were compared to variety standards of M202. The seeds were planted as panicle rows in isolated blocks representing each event. Transgenic plots were sprayed with 1,600 g.a.i./ha of LibertyTM herbicide or not sprayed ("no-spray" plots). Plots with non-
10 transgenic variety standards were not sprayed with LibertyTM. Standard herbicide treatments to control local weeds were applied to all plots.

Transgenic events were tested for yield performance in different locations including California and Puerto Rico (winter nursery).

15

Statistical analysis of the agronomic parameters and ranking statistics of the plant morphology and other non-parametric data were completed to identify the best commercial candidate to compete with the parent variety, M202 and the following rice varieties: M201, M103, Drew, Kaybonnet, Lagrue, Priscilla, Cypress, Bengal,
20 Cocadrie, Jefferson, Madison. GAT-OS1 was the event showing the most utility for producing a range of breeding lines.

Example 3. Characterization of event GAT-OS1

25 a) In-depth molecular and genetic analysis of the locus

Once the GAT-OS1 event was identified as the event in which expression of the transgene as well as overall agronomic performance were optimal, the locus of the transgene was analyzed in detail on a molecular level. This included detailed Southern
30 blot analysis and sequencing of one of the flanking regions of the transgene.

(1) Southern blot analysis using multiple restriction enzymes

Leaf tissue was harvested from transgenic and control plants. Total genomic DNA was isolated from leaf tissue according to Dellaporta et al. (1983, Plant Molecular Biology Reporter, 1, vol.3, p.19-21). The DNA concentration of each preparation was determined by measuring the optical density in a spectrophotometer at a wavelength
5 of 260 nm.

10 μ g of genomic DNA was digested with restriction enzyme in a final reaction volume of 40 μ l, applying conditions proposed by the manufacturer. The time of digestion and/or amount of restriction enzyme were adjusted to ensure complete digestion of the genomic DNA samples without non-specific degradation. After
10 digestion, 4 μ l of loading dye was added to the digested DNA samples, and they were loaded on a 1% agarose gel.

The following control DNAs were also loaded on the gel:

- 15 - a negative control with genomic DNA prepared from a non-transgenic rice plant. This negative control is used to confirm the absence of background hybridization.
- a DNA positive control: With a heterozygous single copy integration of the transgene into the *Oryza sativa* genome, 10 μ g of genomic DNA has the same number of molecule equivalents as \pm 19 picogram of 1501 bp PvuI-HindIII fragment of
20 pB5/35Sbar DNA (*Oryza sativa* diploid genome size: 0.8×10^9 bp). The amount representing one plasmid copy per genome is added to 1 μ g of digested non-transgenic *Oryza sativa* DNA. This reconstitution sample is used to show that the hybridizations are performed under conditions allowing hybridization of the probe with target sequences.

25

Phage Lambda DNA (strain Clind 1 ts 857 Sam 7, Life Technologies) digested with PstI was included as size standard.

30 After electrophoresis, the DNA samples (digested rice genomic DNA, controls and size standard DNA) were transferred to a Nylon membrane by capillary blotting during 12 to 16 hours. The DNA templates used for probe preparation were prepared by restriction digestion of plasmid pB5/35Sbar with EcoRI. This released a 1327 bp DNA fragment that encompasses a relevant part of the transforming DNA (1501 bp

PvuI-HindIII fragment). After purification, the DNA fragment was labeled according to standard procedures, and used for hybridizing to the membrane.

Hybridization was performed under standard stringency conditions: The labeled probe
 5 was denaturated by heating for 5 to 10 minutes in a water bath at 95°C to 100°C and
 chilling on ice for 5 to 10 minutes and added to the hybridization solution (6 X SSC
 (20 X SSC is 3.0 M NaCl, 0.3 M Na citrate, pH 7.0), 5 X Denhardt's (100 X
 Denhardt's = 2% Ficoll, 2% Polyvinyl pyrrolidone, 2% Bovine Serum Albumin), 0.5
 % SDS and 20 µg/ml denatured carrier DNA (single-stranded fish sperm DNA, with
 10 an average length of 120 - 3000 nucleotides). The hybridization was performed
 overnight at 65°C. The blots were washed three times for 20 to 40 minutes at 65°C,
 with the wash solution (2 X SSC, 0.1 % SDS).

The autoradiographs were electronically scanned.

15

The restriction patterns obtained after digestion of GAT-OS1 genomic DNA with
 different restriction enzymes is presented in Figure 1 and summarized in Table 1.

Table 1: Restriction map of GAT-OS1

Lane numbe r	DNA Loaded	Migration of hybridizing DNA fragments between size marker bands		Estimated length of the hybridizing DNA fragments.
		Larger than	Smaller than	
1	Non-transgenic rice	-	-	-
2	Control plasmid DNA - EcoRI			1327 bp
3	GAT-OS1 - NsiI	5077	14057	12 kbp
		5077	11497	7,0 kbp
4	GAT-OS1 - NcoI	2838	4507	3,2 kbp
		2140	2443	2,3 kbp
		1986	2443	2,1 kbp
		805	1093	1,0 kbp

5	GAT-OS1 –	11497	-	14 kbp
	HindIII	5077	14057	13 kbp
6	GAT-OS1 – EcoRV	5077	14057	12 kbp
		4507	5077	4.7 kbp
		2838	4507	2.9 kbp
		1700	1986	1.7 kbp
		1159	1700	1.6 kbp
		805	1159	1.1 kbp
		805	1093	1.0 kbp
		514	805	0.6 kbp
7	GAT-OS1 – EcoRI	1159	1986	1.7 kbp
		1159	1700	1328 bp
		514	805	0.7 kbp
		-	805	0.5 kbp
				other fragments*
8	Lambda DNA - PstI	Not Applicable		Not Applicable

*The digestion of GAT-OS1 with EcoRI always produces at least 2 fragments, and sometimes produces additional fragments. Some of these are attributed to incomplete digestion by EcoRI, which is a known feature of the EcoRI restriction enzyme and is not due to heterogeneity of the starting material

(2) identification of a flanking region

The sequence of one of the regions flanking the inserted transgene in the GAT-OS1 event was determined using the thermal asymmetric interlaced (TAIL-) PCR method as described by Liu et al. (1995, The Plant Journal 8(3):457-463). This method utilizes three nested specific primers in successive reactions together with a shorter arbitrary degenerate (AD) primer so that the relative amplification efficiencies of specific and non-specific products can be thermally controlled. The specific primers were selected for annealing to the border of the transgene and based on their annealing conditions. A small amount (5µl) of unpurified secondary and tertiary PCR

products were analyzed on a 1% agarose gel. The tertiary PCR product was used for preparative amplification, purified and sequenced on an automated sequencer using the DyeDeoxy Terminator cycle kit.

5 TAIL-PCR (PvuI site):

The primers used were:

	Sequence (5' → 3')	Position in pB5/35Sbar
Degenerate primer MDB363	CSg.gNT.gAW.NTA.AWA.C	-----
Primary TAIL MDB424	AAg.gAT.AgT.ggg.ATT.gTg.Cg	3037→3056
Secondary TAIL MDB442	AAT.ggA.ATC.CgA.ggA.ggT.TTC.C	3283→3304
Tertiary TAIL OSA03	gAC.TCT.gTA.TgA.ACT.gTT.CgC	3442→3462

whereby: N=A,C,T or g; S=C or g; W=A or T

10

The fragment amplified using MDB363-YTP054 was ca. 950 bp (3' flank: SEQ ID No. 1). The sequence between bp 1 and bp 603 corresponded to pB5/35Sbar DNA, while bp 604 to bp 1009 comprised plant DNA.

15 (3) genetic analysis of the locus

The genetic stability of the insert was checked by molecular and phenotypic analysis in the progeny plants over several generations.

Southern blot analyses on glufosinate resistant plants of GAT-OS1 rice plants of the T_0 , T_1 , T_2 and T_3 generation were compared and were found to be identical. This proves that the molecular configuration of the transgene in GAT-OS1 containing plants was stable.

The GAT-OS1 event displayed Mendelian segregation for the transgene as a single genetic locus in at least three subsequent generations indicating that the insert is stable.

On the basis of the above results GAT-OS1 was identified as an elite event.

b) Development of diagnostic tools for identity control

The following protocols were developed to identify any rice plant material comprising the elite event GAT-OS1.

(1) GAT-OS1 Elite event Restriction map identification protocol

Rice plants containing the elite event GAT-OS1 can be identified by Southern blotting using essentially the same procedure as described in Example 3 a) (1). Thus rice genomic DNA is 1) digested with at least two, preferably at least 3, for instance with at least 4, more preferably with all of the following restriction enzymes: NsiI, NcoI, HindIII, EcoRV, EcoRI, 2) transferred to nylon membranes and 3) hybridized with the 1327 bp EcoRI fragment of plasmid pB5/35Sbar. If, with respect to each of the restriction enzymes, DNA fragments are identified with the same length as those listed in Table 1, the rice plant is determined to harbor elite event GAT-OS1.

(2) GAT-OS1 Elite event Polymerase Chain Reaction identification protocol

A test run, with all appropriate controls, has to be performed before attempting to screen unknowns. The presented protocol might require optimization for components that may differ between labs (template DNA preparation, Taq DNA polymerase, quality of the primers, dNTP's, thermocycler, etc.)

Amplification of the endogenous sequence plays a key role in the protocol. One has to attain PCR and thermocycling conditions that amplify equimolar quantities of both the endogenous and transgenic sequence in a known transgenic genomic DNA template. Whenever the targeted endogenous fragment is not amplified or whenever the targeted sequences are not amplified with the same ethidium bromide staining intensities, as judged by agarose gel electrophoresis, optimization of the PCR conditions may be required.

10 **Template DNA**

Template DNA is prepared according to Edwards *et al.* (Nucleic Acid Research, 19, p1349, 1991). When using DNA prepared with other methods, a test run utilizing different amounts of template should be done. Usually 50 ng of genomic template DNA yields the best results.

Assigned positive and negative controls

The following positive and negative controls should be included in a PCR run:

20

- Master Mix control (DNA negative control). This is a PCR in which no DNA is added to the reaction. When the expected result, no PCR products, is observed this indicates that the PCR cocktail was not contaminated with target DNA.

25

- A DNA positive control (genomic DNA sample known to contain the transgenic sequences). Successful amplification of this positive control demonstrates that the PCR was run under conditions which allow for the amplification of target sequences.

30

- A wildtype DNA control. This is a PCR in which the template DNA provided is genomic DNA prepared from a non-transgenic plant. When the expected result, no amplification of the transgene PCR product but amplification of the endogenous

PCR product, is observed this indicates that there is no detectable transgene background amplification in a genomic DNA sample.

Primers

5

The following primers, which specifically recognize the transgene and flanking sequence of GAT-OS1 are used:

10

OSA03: 5'-gAC.TCT.gTA.TgA.ACT.gTT.CgC-3' (SEQ ID 2)
(target: P35S)

OSA05: 5'-gTT.CAT.CgA.gTg.gAT.ggC.ACC-3' (SEQ ID 3)
(target: plant DNA)

15

Primers targeting an endogenous sequence are always included in the PCR cocktail. These primers serve as an internal control in unknown samples and in the DNA positive control. A positive result with the endogenous primer-pair demonstrates that there is ample DNA of adequate quality in the genomic DNA preparation for a PCR product to be generated. The endogenous primers used are:

20

OSA01: 5'-gAT.CAg.TgC.Agg.CAA.TAC.Tgg-3' (SEQ ID 4)
(Phospholipase D gene Acc. No. AB001919, 3836→3856)

25

OSA02: 5'-TTC.CTA.ACA.TgT.ggg.TgT.Cg-3' (SEQ ID 5)
(Phospholipase D gene Acc. No. AB001919, 4291→4272)

Amplified fragments

The expected amplified fragments in the PCR reaction are:

30

For primer pair OSA01-OSA02: 457bp (endogenous control)
For primer pair OSA03-OSA05: 522bp (GAT-OS1 Elite Event)

PCR conditions

The PCR mix for 50 μ l reactions contains:

- 5 5 μ l template DNA
 5 μ l 10x Amplification Buffer (supplied with Taq polymerase)
 1 μ l 10 mM dNTP's
 0.75 μ l OSA01 (10pmoles/ μ l)
 0.75 μ l OSA02 (10pmoles/ μ l)
10 2 μ l OSA03 (10pmoles/ μ l)
 2 μ l OSA05 (10pmoles/ μ l)
 0.2 μ l Taq DNA polymerase (5 units/ μ l)
 water up to 50 μ l

- 15 The thermocycling profile to be followed for optimal results is the following:

- 4 min. at 95°C
 Followed by: 1 min. at 95°C
 1 min. at 57°C
20 2 min. at 72°C
 For 5 cycles
 Followed by: 30 sec. at 92°C
 30 sec. at 57°C
 1 min. at 72°C
25 For 22 to 25 cycles
 Followed by: 5 minutes at 72°C

Agarose gel analysis

- 30 Between 10 and 20 μ l of the PCR samples should be applied on a 1.5% agarose gel
 (Tris-borate buffer) with an appropriate molecular weight marker (e.g. 100bp ladder
 PHARMACIA).

Validation of the results

Data from transgenic plant DNA samples within a single PCR run and a single PCR cocktail should not be acceptable unless 1) the DNA positive control shows the expected PCR products (transgenic and endogenous fragments), 2) the DNA negative control is negative for PCR amplification (no fragments) and 3) the wildtype DNA control shows the expected result (endogenous fragment amplification).

Lanes showing visible amounts of the transgenic and endogenous PCR products of the expected sizes, indicate that the corresponding plant from which the genomic template DNA was prepared, has inherited the GAT-OS1 elite event. Lanes not showing visible amounts of the transgenic PCR product and showing visible amounts of the endogenous PCR product, indicate that the corresponding plant from which the genomic template DNA was prepared, does not comprise the elite event. Lanes not showing visible amounts of the endogenous and transgenic PCR products, indicate that the quality and/or quantity of the genomic DNA didn't allow for a PCR product to be generated. These plants cannot be scored. The genomic DNA preparation should be repeated and a new PCR run, with the appropriate controls, has to be performed.

Use of discriminating PCR protocol to identify GAT-OS1

Rice leaf material from plants comprising different transgenic events (samples 1 to 10) was tested according to the above-described protocol. Samples from M202 wildtype and Bengal wildtype were taken as negative controls.

The results of the PCR analysis are illustrated in Figure 2. Samples 10 and 11 (which in fact contained DNA from plants derived from the same event) are recognized as comprising elite event GAT-OS1. All other tested lines do not comprise this elite event.

Example 4. Introgression of GAT-OS1 into preferred cultivars

Elite event GAT-OS1 is introduced by repeated backcrossing into the following cultivars:

- California Temperate Japonicas (such as but not limited to M204, M202, M201, M103)
- 5 - California Tropical Japonicas (such as but not limited to L201, L202)
- Japanese and Korean Temperate Japonicas (such as but not limited to Koshihikari and Milyang)
- Australian Temperate Japonicas (such as but not limited to Millin and Jarrah)
- Mediterranean Temperate Japonicas (such as but not limited to Ballila, Arborio)
- 10 - Chinese Indicas (such as but not limited to Guichao, Congui 314, Teqing)
- Southern United State Tropical Japonicas, long grain (such as but not limited to Drew, Cypress, Jefferson, Priscilla, Cocadrie)
- Southern United State Tropical Japonicas, medium grain (such as but not limited to Bengal, Mars, Brazos, Mercury)
- 15 - South American Tropical Japonicas, long grain (such as but not limited to El Paso 144, IRGA 409)
- Far Eastern basmati and jasmine types (Kasmir, Kwao Dak Mali)
- African javanica types (bulu rices)

- 20 It is observed that the introgression of the elite event into these cultivars does not significantly influence any of the desirable phenotypic or agronomic characteristics of these cultivars (no linkage drag) while expression of the transgene, as determined by glufosinate tolerance, meets commercially acceptable levels. This confirms the status of event GAT-OS1 as an elite event.

As used in the claims below, unless otherwise clearly indicated, the term "plant" is intended to encompass plant tissues, at any stage of maturity, as well as any cells, tissues, or organs taken from or derived from any such plant, including without
30 limitation, any seeds, leaves, stems, flowers, roots, single cells, gametes, cell cultures, tissue cultures or protoplasts.

Seed comprising elite event GAT-OS1 was deposited as GAT-OS1 at the ATCC under number: ATCC 203353.

31
CLAIMS

- 1) A transgenic, glufosinate tolerant rice plant, cell, tissue or seed, which is characterized in that:
- 5 a) the genomic DNA of said plant, cell, tissue or seed is capable of yielding at least two sets of restriction fragments, wherein said sets of restriction fragments are selected from the group of:
- 10 i) one set of NsiI fragments comprising at least: one fragment with a length between 5077 and 14057 bp, and one fragment with a length between 5077 and 11497 bp;
- ii) one set of NcoI fragments comprising at least: one fragment with a length between 2838 and 4507 bp, one fragment with a length between 2140 and 2443 bp, and one fragment with a length between 1986 and 2140 bp;
- 15 iii) one set of HindIII fragments comprising at least: one fragment with a length of more than 11497 bp, and one fragment with a length between 5077 and 14057 bp;
- iv) one set of EcoRV fragments comprising at least: one fragment with a length between 1700 and 1986 bp, one fragment with a length between 1159 and 1700 bp, and one fragment with a length between 805 and 1093 bp;
- 20 v) one set of EcoRI fragments comprising at least: one fragment with a length between 1159 and 1986 bp, and one fragment with a length between 1159 and 1700 bp;
- 25 wherein each of said restriction fragments is capable of hybridizing under standard stringency conditions, with the 1327 bp fragment obtainable by EcoRI digestion of the plasmid having the nucleotide sequence of SEQ ID No 6;
- and/or,
- 30 b) a DNA fragment of between 490 and 550 bp, preferably of about 522 bp, can be amplified from the genomic DNA of said plant, cell, tissue or seed using a polymerase chain reaction with two primers having the nucleotide sequence of SEQ ID No 2 and SEQ ID No 3 respectively.

- 2) The plant, cell, tissue or seed of claim 1, which is characterized in that a DNA fragment of between 490 and 550 bp, preferably of about 522 bp can be amplified from genomic DNA of said plant, cell, tissue or seed using a polymerase chain reaction with two primers having the nucleotide sequence of SEQ ID No 2 and SEQ ID No 3 respectively.
- 3) The plant, cell, tissue or seed of claim 1, which is characterized in that genomic DNA of said plant, cell, tissue or seed is capable of yielding at least two sets of restriction fragments, wherein said sets of restriction fragments are selected from the group of:
- i) one set of NsiI fragments comprising at least: one fragment with a length between 5077 and 14057 bp, and one fragment with a length between 5077 and 11497 bp;
 - ii) one set of NcoI fragments comprising at least: one fragment with a length between 2838 and 4507 bp, one fragment with a length between 2140 and 2443 bp, and one fragment with a length between 1986 and 2140 bp;
 - iii) one set of HindIII fragments comprising at least: one fragment with a length of more than 11497 bp, and one fragment with a length between 5077 and 14057 bp;
 - iv) one set of EcoRV fragments comprising at least: one fragment with a length between 1700 and 1986 bp, one fragment with a length between 1159 and 1700 bp, and one fragment with a length between 805 and 1093 bp;
 - v) one set of EcoRI fragments comprising at least: one fragment with a length between 1159 and 1986 bp, and one fragment with a length between 1159 and 1700 bp;
- wherein each of said restriction fragments is capable of hybridizing, under standard stringency conditions, with the 1327 bp fragment obtainable by EcoRI digestion of the plasmid having the nucleotide sequence of SEQ ID No 6.

- 4) The plant, cell, tissue or seed of claim 3, which is characterized in that genomic DNA of said plant, cell, tissue or seed is capable of yielding at least three sets of restriction fragments selected from said group.
- 5) The plant, cell, tissue or seed of claim 4, which is characterized in that genomic DNA of said plant, cell, tissue or seed is capable of yielding at least four sets of restriction fragments selected from said group.
- 6) The plant, cell, tissue or seed of claim 5, which is characterized in that genomic DNA of said plant, cell, tissue or seed is capable of yielding the five sets of restriction fragments selected from said group.
- 7) The plant, cell, tissue or seed of any one of claims 3 to 6 which is further characterized in that a DNA fragment of between 490 and 550 bp, preferably of about 522 bp, can be amplified from genomic DNA of said plant, cell, tissue or seed using a polymerase chain reaction with two primers having the nucleotide sequence of SEQ ID No 2 and SEQ ID No 3 respectively.
- 8) A plant which is grown from a seed deposited at the ATCC under number ATCC 203353.
- 9) A cell or tissue of the plant of claim 8.
- 10) A seed deposited at the ATCC under number 203353.
- 11) A transgenic, glufosinate tolerant rice plant of any one of claims 1 to 7 which is obtainable by propagation of, and/or breeding with, a rice plant grown from a seed deposited at the ATCC under number 203353.
- 12) A process for cultivating rice plants which comprises growing plants of any one of claims 1 to 8.

- 13) The process of claim 12 which further comprises applying a herbicide with glufosinate as an active ingredient to the cultivated rice plants.
- 14) A process for breeding rice which comprises a crossing with a plant of any one of claims 1 to 8.
- 15) A process for producing a transgenic cell of a rice plant which comprises inserting a recombinant DNA molecule into a part of the chromosomal DNA of a rice cell characterized by the sequence of SEQ ID No 7.
- 16) A transgenic cell of a rice plant obtainable by the method of claim 15.
- 17) A process for producing a transgenic rice plant which comprises inserting a recombinant DNA molecule into a part of the chromosomal DNA of a rice cell characterized by the sequence of SEQ ID No 7, and regeneration of a rice plant from the transformed rice cell.
- 18) A transgenic rice plant obtainable by the method of claim 17.
- 19) A method for identifying a transgenic plant, or cells or tissues thereof, comprising the elite event GAT-OS1, which method comprises establishing one or both of the following characteristics.
- a) the genomic DNA is capable of yielding at least two of the sets of the restriction fragments, wherein selected from the group of:
- i) one set of NsiI fragments wherein one fragment has a length between 5077 and 14057 bp and one has a length between 5077 and 11497 bp;
- ii) one set of NcoI fragments wherein one fragment has a length between 2838 and 4507 bp, one fragment has a length between 2140 and 2443 bp, one fragment has a length between 1986 and 2140 bp, and one fragment has a length between 805 and 1093 bp;
- iii) one set of HindIII fragments wherein one fragment has a length of more than 11497 bp, and one fragment has a length between 5077 and 14057 bp;

- iv) one set of EcoRV fragments wherein one fragment has a length between 5077 and 14057 bp, one fragment has a length between 4507 and 5077 bp, one fragment has a length between 2838 and 4507 bp, one fragment has a length between 1700 and 1986 bp, one fragment has a length between 1159 and 1700 bp, one fragment has a length between 805 and 1159 bp, one fragment has a length between 805 and 1093 bp, and one fragment has a length of between 514 and 805 bp;
- v) one set of EcoRI fragments, wherein one fragment has a length between 1159 and 1986 bp, one fragment has a length between 1159 and 1700 bp, one has a length between 514 and 805 bp, and one fragment has a length of less than 805 bp;

wherein each of the restriction fragments is capable of hybridizing under standard stringency conditions, with the 1327 bp fragment obtainable by EcoRI digestion of the plasmid having the nucleotide sequence of SEQ ID No. 6; and/or

- b) the genomic DNA of the plant, cell, tissue or seed can be used to amplify a DNA fragment of about 522 bp using a polymerase chain reaction with two primers having the nucleotide sequence of SEQ ID No. 2 and SEQ ID No. 3 respectively.
- 20) The method of claim 19, which comprises establishing whether the genomic DNA of the transgenic plant, or its cells or tissues is capable of yielding all five of said restriction fragments or sets of restriction fragments.
- 21) The method of claim 19, which comprises establishing whether the genomic DNA of the transgenic plant, or its cells or tissues can be used to amplify a DNA fragment of about 522 bp using a polymerase chain reaction with two primers having the nucleotide sequence of SEQ ID No. 2 and SEQ ID No. 3 respectively.
- 22) A kit for identifying a transgenic plant, its cells or tissues comprising the MS-B2 elite event, said kit comprising at least two PCR probes, one of which

recognizes a sequence within the foreign DNA of GAT-OS1, the other which recognizes a sequence within the 3' or 5' flanking region of GAT-OS1.

23) The kit of claim 22, said kit comprising the PCR probes having the nucleotide sequence of SEQ ID No. 2 and SEQ ID No. 3 respectively.

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Figure 1

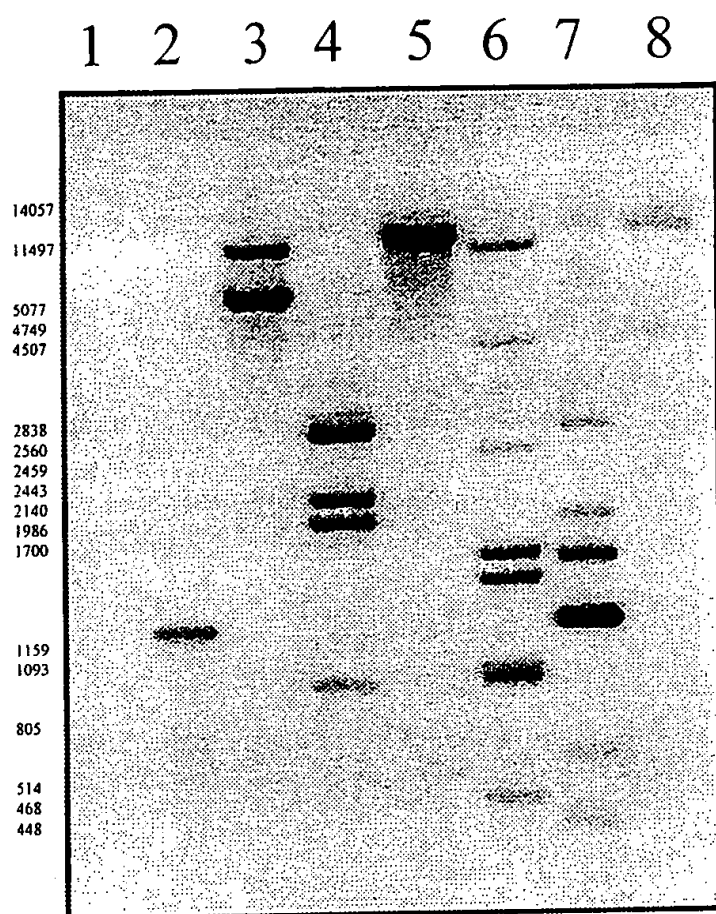
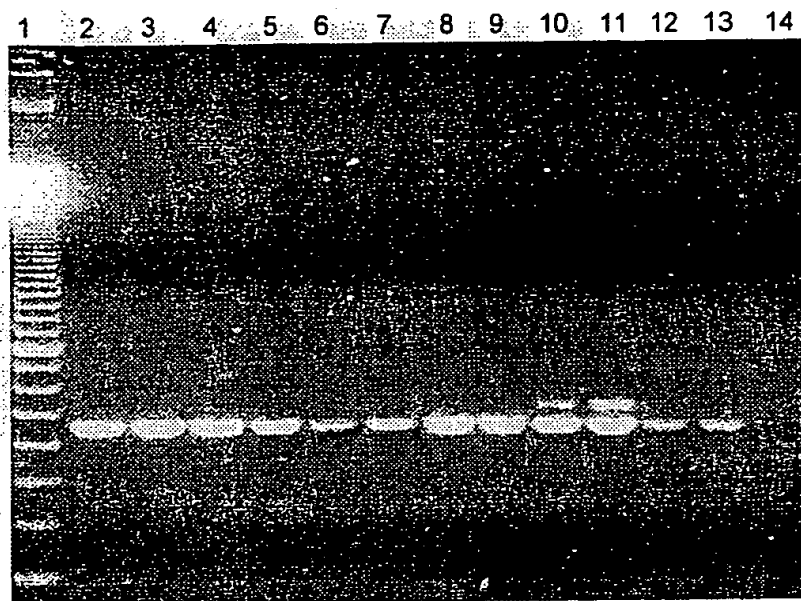


Figure 2



INTERNATIONAL SEARCH REPORT

International application No.
PCT/US99/25666

A. CLASSIFICATION OF SUBJECT MATTER

IPC(7) : C12N 15/00, 15/29, 15/82; A01H 3/00, 4/00, 5/00

US CL : Please See Extra Sheet.

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 435/419, 468, 320.1; 536 23.5, 23.6, 24.1; 800/278, 288293, 295, 298, 300, 320.2

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

EAST, CABA, CAPLUS, GENBANK, BIOSIS

search terms: rice, BAR, glufosinate, phosphinothricin, biaphalos, biolistic, transform, transgenic

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	US 5,641,664 A (D'HALLUIN et al.) 24 June 1997, entire document.	1-23N
Y	DEKEYSER et al. Evaluation of Selectable Markers for Rice Transformation. Plant Physiol. 1989. Vol. 90, pages 217-223, see entire document.	1-23
Y	US 5,646,024 A (LEEMANS et al.) 08 July 1997, see entire document.	1-23



Further documents are listed in the continuation of Box C.



See patent family annex.

* Special categories of cited documents:

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L document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

O document referring to an oral disclosure, use, exhibition or other means

P document published prior to the international filing date but later than the priority date claimed

T

later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

X

document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

Y

document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

A

document member of the same patent family

Date of the actual completion of the international search

20 MARCH 2000

Date of mailing of the international search report

06 APR 2000

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INTERNATIONAL SEARCH REPORT

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A. CLASSIFICATION OF SUBJECT MATTER:

US CL :

435/419, 468, 320.1; 536 23.5, 23.6, 24.1; 800/278, 288293, 295, 298, 300, 320.2

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